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Pre-Transplant IgG Reactivity to Apoptotic Cells Correlates with Late kidney Allograft Loss

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Abstract

Pre-existing serum antibodies have long been associated with graft loss in transplant candidates. While most studies have focused on HLA-specific antibodies, the contribution of non-HLA-reactive antibodies has been largely overlooked. We have recently characterized monoclonal antibodies secreted by B cell clones derived from kidney allograft recipients with rejection that selectively bind to apoptotic cells. Here, we assessed the presence of such antibodies in pre-transplant serum from 300 kidney transplant recipients and examined their contribution to the graft outcomes. Kaplan-Meier survival analysis revealed that patients with high pre-transplant IgG reactivity to apoptotic cells had a significantly increased rate of late graft loss. The effect was only apparent after approximately 1 year post-transplant. Moreover, the association between pre-transplant IgG reactivity to apoptotic cells and graft loss was still significant after excluding patients with high reactivity to HLA. This reactivity was almost exclusively mediated by IgG1 and IgG3 with complement fixing and activating properties. Overall, our findings support the view that IgG reactivity to apoptotic cells contribute to pre-sensitization. Taking these antibodies into

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consideration alongside anti-HLA antibodies during candidate evaluation would likely improve the transplant risk assessment.

Keywords

Anti-apoptotic cell antibodies; sensitization; apoptotic cells; kidney transplantation; graft loss; complement

Introduction

Pre-sensitization has been a major limitation to solid organ transplantation for decades. Candidate recipients with pre-existing antibodies to potential donor grafts have a higher risk of rejection and eventually graft loss (1–6). It is commonly accepted that these antibodies are either naturally pre-formed or had developed after exposure to allogeneic antigens occurring during pregnancy, blood transfusion or previous allografts. In ABO compatible donor-recipient pairs, sensitizing antibodies are primarily IgG reactive to human leukocyte antigen (HLA). However, a number of observations suggest that non-HLA reactive antibodies also contribute to pre-sensitization and may influence the overall graft outcome (7–9). Cases of early humoral rejection in the absence of detectable donor-specific antibodies (DSA) have also been reported (10, 11). In a landmark collaborative transplant study, Opelz and colleagues revealed the association between high panel reactive antibodies (PRA) before transplantation and late graft loss in recipients of kidney transplants from HLA identical siblings (12). Since the donors and recipients shared both HLA loci, the PRA impact on graft survival could not be attributed to donor specific HLA antibodies. Additional studies support a contribution of non-HLA antibodies to pre-sensitization (7, 13). More specifically, serum IgG reactivity to autoantigens such as cardiac myosin, vimentin, collagen, oxidized lipids and LG3 has been associated with increased rejection rates and reduced graft survival (14–23).

Natural antibodies are distinct antibodies that develop without any evidence of immunization (24). An important characteristic of natural antibodies is their capacity to react to apoptotic cells (25–28). These antibodies are primarily IgM, although IgG have also been detected in various pathological conditions, indicating class switch recombination (CSR) of the producing B cells. Despite their essential role in health and diseases, anti-apoptotic cell antibodies have seldom been examined in the context of human transplantation.

In previous studies, we isolated a number of B cell clones secreting antibodies reactive to apoptotic cells from a kidney transplant recipient with antibody mediated rejection (AMR) (29). More generally, we also observed elevated IgG reactivity to apoptotic cells in kidney transplant recipients experiencing AMR compared to patients with stable graft function (30). Collectively, these findings alluded to a contribution of anti-apoptotic cell IgG to the pathophysiology of graft rejection. In this study, we examined the contribution of serum IgG reacting to apoptotic cells to pre-sensitization and graft outcome on a large cohort of patients who received a kidney transplant at Massachusetts General Hospital (MGH) between 1999 and 2007.

Materials and Methods

Patient characteristics and sample collection

The collection of all specimens used in this study was approved by MGH internal review board. The patient group consisted of 300 non-consecutive kidney transplant recipients who received a kidney transplant at MGH between May 1999 and July 2007 and whose pre-transplant serum specimens were available. Patients with pre-transplant DSA were excluded in this study. All serum specimens were collected prior to transplantation as part of the patients' standard clinical care. Serum samples collected from 20 healthy subjects were used as control in this study. The baseline characteristics of all patients included in this study are summarized as Table 1. Forty six of the 300 patients included in our study lost their grafts and returned to dialysis. For 42 of these patients, the cause of graft loss was based on pathological changes seen in biopsy specimens. For the remaining 4 patients, the cause of graft loss was determined by clinical criteria and serological tests.

Serum IgG purification

Serum IgG were purified from patients specimens using the Melon Gel IgG Purification Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Briefly, serum samples were diluted 1:10 in a dilution buffer and incubated with a resin that retains non-IgG immunoglobulin as well as other abundant serum proteins.

Assessment of reactivity to apoptotic cells

We used flow cytometry to assess the reactivity of serum IgM, IgG and purified IgG to apoptotic Jurkat cells in samples collected pre-transplant from 300 patients who received a kidney transplant at MGH between 1999 and 2007 as well as 20 control healthy subjects. In these experiments, human Jurkat T cell leukemia cells were cultured overnight with 200ng/ml of anti-FAS antibody (clone CH11, Millipore, Billerica, MA) or exposed to UV light (240×10^{-3} J) to induce apoptosis using a UV stratalinker 2400 (Stratagene, Santa Clara, CA). Then 1×10^6 apoptotic Jurkat T cells were incubated for 30 minutes at 37°C with 100µl serum IgM and IgG samples diluted 1:5 in PBS or 60µl purified IgG samples diluted 1:2 in PBS. After two washes in 3ml PBS at 4°C, samples were incubated with FITC-conjugated anti-IgM or anti-IgG F(ab')₂ secondary antibodies, respectively (Invitrogen, Camarillo, CA) for 30 minutes at 4°C. After two additional washes in 3ml PBS at 4°C, cells were acquired using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) after gating on apoptotic cells. To avoid inter-experiment variations, all samples were assessed at the same time in the same experiment using the same instrument settings. Results are reported as log₂ values of the mean fluorescence intensity (MFI) of positive cells. A titration experiments carried out with purified IgG samples from 4 patients with highest IgG reactivity to apoptotic cells is reported in Figure S1.

Generation of HLA class I negative Jurkat cells

Supernatants containing packaged pLKO.1 lentiviral vector containing a shRNA specific for the human β₂-microglobulin was obtained from Dr. Roberto Bellucci (Dana-Farber Cancer Institute). Preparation of the lentiviral vector is described elsewhere (31). Jurkat cells were

transduced with virus supernatants and polybrene at 8 μ g/ml (Millipore) two times and selected with puromycin for 24 hours after the second transduction. HLA class I negative cells were then purified from transduced cells by sorting. This operation was repeated three times until homogeneous HLA class I negative was obtained.

Quantification of IgM and IgG concentration

Serum IgM and IgG in patients and healthy subjects were quantified using a Cytometric Bead Array kit (BD Biosciences, San Jose, CA). Briefly, capture beads were incubated with 50 μ l samples diluted at 1:2,500 for IgM, 1:100,000 for IgG and 1:10,000 for purified IgG with dilution buffer for 1h at room temperature. After 1 wash in 1ml wash buffer, 50 μ l mixed PE detection reagent was added in each sample and incubated for 2h at room temperature. After 1 wash in 1 ml wash buffer, beads were resuspended in 300 μ l wash buffer and acquired using an Accuri C6 flow cytometer (BD Biosciences). Immunoglobulin concentrations were calculated using FCAP Array v3.0 software (BD Biosciences). IgG subclasses (IgG1 ~ IgG4) in patients before and after IgG purification were quantified by ELISA using Human IgG Subclass Profile Kit (Invitrogen, Camarillo, CA) according to the manufacturer's instruction. Briefly, 50 μ l serum samples diluted at 1:2500 were incubated for 30 minutes at room temperature with anti-human IgG1~ IgG4 subclasses specific antibodies, respectively. Plates were washed 4 times in wash buffer, peroxidase anti-human IgG solution was added and incubated for 30 minutes at room temperature, after 4 washes, developed using 3, 3', 5, 5'-tetramethylbenzidine. Optical density was measured at 450 nm.

Detection of reactivity to HLA and MICA molecules

The reactivity of patients' serum to HLA Class I, HLA Class II, or MICA was assessed using beads coated with mixed HLA molecules (LABScreen Mixed, One Lambda, Los Angeles, CA). Antibodies reactive to beads were detected with an anti-IgG (One Lambda) PE-conjugated secondary antibody on a Luminex 200 apparatus (Luminex, Austin, TX). A MFI of 1,000 was arbitrarily used as a cutoff value.

Serum IgG subclass reactivity to apoptotic cells

Apoptotic Jurkat (1×10^6 cells) cells were incubated for 30 minutes at 37°C with 60 μ l IgG purified from the 50 most reactive patient serum specimens diluted 1:2. After two washes in 3ml PBS at 4°C, samples were incubated with PE-conjugated anti-IgG1, IgG2, IgG3, IgG4 secondary antibodies for each patient, respectively (Clone 4E3, HP6002, HP6050, HP6025, Southern Biotech, Birmingham, AL) at 4°C for 30 minutes. After two washes, cells were acquired using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) after gating on apoptotic cells.

C4d binding assay

Apoptotic Jurkat cells (0.5×10^6 cells) were incubated for 30 minutes at 37°C with 60 μ l purified serum IgG (see above) diluted 1:2. Human serum from a healthy subject diluted 1:100 in HBSS was then added as a source of complement and incubated for 15 minutes at 37°C. After 2 washes in PBS, cells were incubated for 30 minutes at 4°C with an anti-C4d antibody (Quidel, San Diego, CA), washed twice in PBS, and then incubated for 30 minutes

at 4°C with a FITC-conjugated anti-mouse IgG secondary antibody (BD Biosciences). After 2 final washes at 4°C, C4d binding was measured after gating on apoptotic cells on an Accuri C6 flow cytometer (BD Biosciences).

Statistical analysis

Mann-Whitney tests were performed to compare IgM or IgG reactivity to apoptotic cells between all subgroups of patients and healthy subjects. We used Spearman correlation tests to determine the association between IgG1 and IgG3 reactivity to apoptotic cells and C4d binding on target cells, between IgG concentration before and after purification, between IgG reactivity to apoptotic cells and patient age and between IgG reactivity to wild type Jurkat cells and reactivity to class I negative Jurkat cells. Kidney graft loss post transplant was reported as death censored graft loss. Graft-survival rates were computed using the Kaplan-Meier method and groups were compared by the log-rank test. Cox models were used to adjust the effects of IgG reactivity to apoptotic cells for potentially confounding factors including reactivity to HLA antibodies, sex, age, race, calculated panel reactive antibody (cPRA), delayed graft function (DGF), HLA mismatch, cold ischemia time, induction therapy and living donor transplants. Hazard ratio and 95% confidence interval are provided as measures of strength of association and precision, respectively. All tests were two-sided and a *P*-value of < 0.05 was considered to be statistically significant. General data analysis was conducted using SAS Analytics Software (SAS Institute Inc, Cary, NC) and GraphPad Prism (GraphPad Software 4.0, San Diego, CA).

Results

Serum reactivity to apoptotic cells before transplantation

In order to evaluate the contribution of anti-apoptotic cell antibodies to pre-sensitization, we assessed pre-transplant serum IgG reactivity to apoptotic cells in 300 kidney transplant recipients as well as 20 healthy controls. We had previously observed that the binding of IgG to apoptotic cells can be masked by serum proteins (30). We thus purified serum IgG before assessing their reactivity to apoptotic cells. As shown in Figure 1, purified IgG reactivity to apoptotic cells was significantly higher in pre-transplant serum compared to healthy subjects (*P* = 0.011). Comparable results were obtained when assessing non purified serum IgG reactivity to apoptotic cells (Figure S2). In contrast, no significant difference in IgM reactivity to apoptotic cells was observed between pre-transplant patients and healthy subjects (*P* = 0.922, Figure S2).

We next verified that the difference observed in reactivity to apoptotic cells between the two groups was not solely due to serum immunoglobulin levels. As illustrated in Figure S3A, concentrations of serum IgM, IgG as well as purified IgG were not significantly different between pre-transplant patients and healthy subjects (*P* = 0.900, *P* = 0.665, *P* = 0.420, respectively). Additionally, concentrations of purified IgG were comparable to that of unpurified serum IgG concentrations in all 300 pre-transplant patients (*P* < 0.001, Figure S3B).

We then examined whether pre-transplant IgG reactivity to apoptotic cells was associated with discrete patient characteristics. We observed a positive correlation between age and purified IgG reactivity to apoptotic cells ($P = 0.024$, not shown). However, reactivity of purified IgG to apoptotic cells did not appear to significantly correlate with sex, race, donor, etiology of kidney failure, previous transplants or history of blood transfusions. In addition, we did not observe any significant difference between patients with autoimmune diseases (including primary focal segmental glomerulosclerosis, IgA nephropathy, type I diabetes, systemic lupus erythematosus and immune complex diseases) and patients with non-autoimmune diseases (Figure S4).

Serum reactivity to HLA class I negative Jurkat cells and viable Jurkat cells

To ensure that reactivity to apoptotic Jurkat cells was not due to the recognition of HLA class I, we generated class I negative Jurkat cells through β -2 microglobulin knockdown using shRNA transfection to use as target (Figure S5A). As shown in Figure S5B, the binding of purified IgG to apoptotic class I negative cells is comparable to that of wild type Jurkat cells in the 39 patients with high IgG reactivity to HLA class I (MFI > 1000), ($r = 0.874$, $P < 0.001$), indicating that these antibodies recognize other antigenic structures than HLA on apoptotic cells. Lastly, as illustrated in Figure S6A for 3 representative pre-transplant samples, no reactivity was detected on viable Jurkat cells.

Pre-transplant purified IgG reactivity to apoptotic cells and kidney graft survival

The mean duration of follow-up for all patients included in this retrospective study was 81.2 ± 35.3 months. Forty-six patients lost their grafts and returned to dialysis due to various complications. The causes of graft loss are reported in Table 2. As depicted in Figure 2A, these patients had significantly higher purified IgG reactivity to apoptotic cells before transplantation compared to those with functioning graft ($P < 0.001$). In contrast, pre-transplant IgG reactivity to viable cells was not significantly different between patients with functioning graft and patients who experienced graft loss ($P = 0.634$, Figure S6B). Remarkably, among the 46 patients who lost their grafts, pre-transplant purified IgG reactivity to apoptotic cells was significantly increased in those whose graft loss was attributed to AMR compared to patients with other causes of graft loss ($P = 0.033$, Figure 2B).

Figure 3A reports the death with functioning graft censored Kaplan-Meier survival outcome for patients with pre-transplant purified IgG reactivity to apoptotic cells above or below the median value ($P = 0.002$). We next separated the patients into 4 groups according to their reactivity to apoptotic cells: below the 1st quartile, between the 1st quartile and the 2nd quartile, between the 2nd quartile and the 3rd quartile or above the 3rd quartile value. As shown in this figure, the graft survival rate was significantly different between these groups ($P < 0.001$, Figure 3B). Patients with pre-transplant purified IgG reactivity to apoptotic cells above the 3rd quartile experienced the worst outcome while patients whose purified IgG reactivity to apoptotic cells was below the 1st quartile value had the highest graft survival rate. The influence of IgG reactivity to apoptotic cells on graft survival was only noticeable after approximately 1 year post-transplantation as apparent in the graft survival curve.

None of the 300 patients included in this study had detectable DSA at time of transplant. However, we assessed the overall pre-transplant serum reactivity to mixed HLA class I, II and MICA by Luminex in order to evaluate its effect on the graft outcome. Results showed that pre-existing reactivity to HLA class II and MICA but not HLA class I above a cutoff value of 1000 MFI was associated with decreased graft survival ($P = 0.039$, $P = 0.006$, $P = 0.539$, respectively; Figure 4A). We next investigated whether reactivity to apoptotic cells still correlated with graft loss when patients with reactivity to HLA class I, class II or MICA above 1000 MFI were excluded. As shown in Figure 4B, pre-transplant IgG reactivity to apoptotic cells still affected graft outcome in patients with low reactivity to HLA class I, class II and MICA ($P = 0.003$, $P = 0.003$, $P = 0.023$, respectively). Conversely, we examined whether reactivity to HLA class I, class II and MICA influenced the graft outcome in patients with IgG reactivity to apoptotic cells below the median value. As depicted in Figure 4C, pre-existing HLA class I, class II and MICA reactive antibodies did not correlate with graft loss in patients with IgG reactivity to apoptotic cells below the median value ($P = 0.794$, $P = 0.091$, $P = 0.665$, respectively). Of note, the number of patients included in this latter analysis was limited. To rule out the possibility that the risk-elevation for graft loss with increased levels of pre-transplant IgG reactivity to apoptotic cells was an artifact due to confounding with other factors including other IgG reactivities, sex, age, race, cPRA, DGF, HLA mismatch, cold ischemia time, induction therapy and living donor transplants, we carried out a Cox proportional hazards analysis including these covariates. The results confirmed that IgG reactivity to apoptotic cells as an explanatory factor remained significantly associated to graft loss even when the reactivity to HLA class I, class II and MICA as well as other variables were included and adjusted for in the statistical model (Table 3). In additional Cox models not shown, the hazard ratio for IgG reactivity to apoptotic cells remained constant throughout post-transplant follow-up time.

Subclasses of serum IgG reactive to apoptotic cells

The activation of the complement pathway is a primary effector function of antibodies implicated in graft failure (32–34). We investigated whether IgG reactive to apoptotic cells could also activate complement. We first used distinct secondary antibodies in flow cytometry experiments to determine the subclasses of IgG reactive to apoptotic cells. These experiments were conducted using IgG purified from the 50 most reactive serum specimens. For all specimens the purified serum IgG reactivity to apoptotic cells was almost exclusively mediated by complement fixing IgG1 or IgG3 subclasses or a combination of the two (Figure 5). Since only few samples showed IgG4 or IgG2 reactivity to apoptotic cells, we verified that our purification procedure did not eliminate these two IgG subclasses. As shown in Figure S7, the concentrations of all four IgG subclasses were comparable before and after IgG purification. We also tested the reactivity of all anti-IgG subclass secondary antibodies used in these assays on peripheral blood B cells from 2 healthy subjects. A distinct positive subset was detectable for each anti-IgG subclass as depicted in Figure S8.

IgG1 and IgG3 reactive to apoptotic cells activate complement

The implication of the complement pathway in graft dysfunction is commonly revealed by the deposition of C4d into the allograft tissue (35–38). We used an in vitro assay to assess whether IgG reactive to apoptotic cells purified from pre-transplant serum specimens have

the capacity to activate complement, resulting in the deposition of C4d on target cells (Figure S9). As expected, purified IgG comprising primarily complement fixing IgG1 and IgG3 had the capacity to activate complement. Moreover, we observed a strong association between IgG1 and IgG3 reactivity to apoptotic cells and C4d deposition on target cells ($P < 0.001$ and $P = 0.005$, respectively; Figure 6).

Discussion

Pre-sensitization has been a long-standing limitation to solid organ transplantation. Until now, pre-sensitization has been explained by the presence of allospecific antibodies reactive to donor HLA or ABO blood group antigens. Our retrospective studies conducted on a relatively large series of patients are the first to implicate IgG reactive to apoptotic cells in pre-sensitization. The observation that pre-transplant anti-apoptotic cell IgG levels correlate with long-term graft loss even after exclusion of patients with pre-existing reactivity to HLA (MFI>1000), highlights the important value of these antibodies in kidney transplantation. Further, the association between serum reactivity to apoptotic cells and AMR even suggests an active role in this form of rejection. It is remarkable that the effect on graft survival was only visible after approximately 1 year post-transplantation as noticeable in the graft survival curve. This observation seems to match that made by Opelz and colleagues who examined the predictive value of pre-transplant PRA on graft survival in recipients of kidney transplants from HLA identical siblings (12). In this study, the effect of high PRA measurements on graft survival was only apparent after one year post-transplant. Our results suggest that anti-apoptotic cell IgG may have contributed to sensitization in these patients. The delayed effect is difficult to interpret. Immune events occurring early post-transplant and resulting from pre-existing anti-apoptotic cell antibodies may polarize the global host response to the graft and significantly influence the late outcome. Another hypothesis is that patients with elevated serum reactivity to apoptotic cells are immunologically more “sensitive” than those with lower pre-transplant serum reactivity. “Sensitive” patients would have an increased probability of complications, resulting in a higher rate of graft loss. Under this hypothesis, the pre-transplant serum reactivity would be a surrogate marker of “immune sensitivity” without any direct relation of cause and effect between pre-transplant antibodies and graft loss.

The generation of de novo DSA and non donor-specific antibodies (NDSA) post-transplant would also provide valuable information regarding the development of immunity to the organ graft in these patients. Unfortunately, our collection of post-transplant serum specimens was limited and we could not establish whether elevated pre-transplant IgG reactivity to apoptotic cells correlated with the development of de novo anti-HLA antibodies especially DSA for these patients. This lack of longitudinal follow-up is a significant limitation to our study. Additional studies will be required to address this point.

The origin of IgG reactive to apoptotic cells in patients with end-stage kidney disease is unclear. While “natural” IgM have been described in animals and humans for decades, class switched IgG bearing the same reactivity have only been reported in the context of certain autoimmune diseases such as systemic lupus erythematosus and more recently AMR (39, 40). Since they share the same distinctive reactivity, both natural IgM and anti-apoptotic cell

IgG are likely produced by the same “innate” B cells that underwent class switch recombination (CSR). The exact cause for innate B cell CSR in kidney transplant candidates is unknown. Long-term hemodialysis treatments which most of the transplant candidates in our study underwent are often accompanied by an increase in inflammatory markers (41, 42). Likewise, several chronic kidney diseases have been associated with heightened levels of systemic inflammation that may have contributed to the generation of anti-apoptotic cell IgG (43).

It is still unclear as to what antigens are recognized by these antibodies at the surface of apoptotic cells. In a previous study, we reported the characterization of monoclonal antibodies reactive to apoptotic cells established from a patient with AMR (30). These monoclonal antibodies were all polyreactive, i.e. they reacted to multiple antigens as diverse as DNA or lipopolysaccharide. We also found that 2 of 6 polyreactive antibodies bound equally to phosphatidylserine and lysophosphatidylcholine. Both antigens are known immunogenic structures exposed at the surface of apoptotic cells. These results indicate that polyreactive antibodies can recognize distinct antigens on apoptotic cells.

The fact that anti-apoptotic cell IgG in our series are almost exclusively composed of complement fixing IgG1 and IgG3 is novel and intriguing. The reason for such a restriction is unclear. The preferential switch to IgG1 and IgG3 may be driven by the type of stimulation received by B cells reactive to apoptotic cells. It is possible that apoptotic cells deliver concomitant signals through the B cell receptor (BCR) as well as through co-stimulatory molecules and Toll-like receptor (TLR) directing CSR towards these 2 IgG subclasses. It is noteworthy however that both IgG1 and IgG3 have complement activating properties. Late allograft failures often involve complement activation, as revealed by the deposition of C4d on the graft tissue. IgG1 and IgG3 appear to play a dominant role in this process (44–47). Along these lines, our findings support the view that IgG1 and IgG3 reactive to apoptotic cells participate in the overall inflammatory response to the allograft and eventually contribute to graft loss.

Results presented in this report shed a new light on anti-apoptotic cell IgG as important contributors to pre-sensitization along with allospecific antibodies. The association between elevated levels of these antibodies and the transplant outcome even suggests a direct physiological role in graft destruction. Our findings have significant implications for the assessment of transplant candidates. Taking these IgG into consideration in addition to anti-HLA antibodies during pre-transplant evaluation would likely improve the risk assessment of the graft outcome. Moreover, patients with high reactivity to apoptotic cells pre-transplant could also possibly benefit from desensitization treatments. Prospective studies are now warranted to extend our observations to additional transplant recipients and further corroborate the involvement of anti-apoptotic cell IgG in the host response to kidney allografts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HLA	human leukocyte antigen
DSA	donor-specific antibodies
PRA	panel reactive antibodies
CSR	class switch recombination
AMR	antibody mediated rejection
MFI	mean fluorescence intensity
cPRA	calculated panel reactive antibody
DGF	delayed graft function
NDSA	nondonor-specific antibodies
BCR	B cell receptor
TLR	Toll-like receptor

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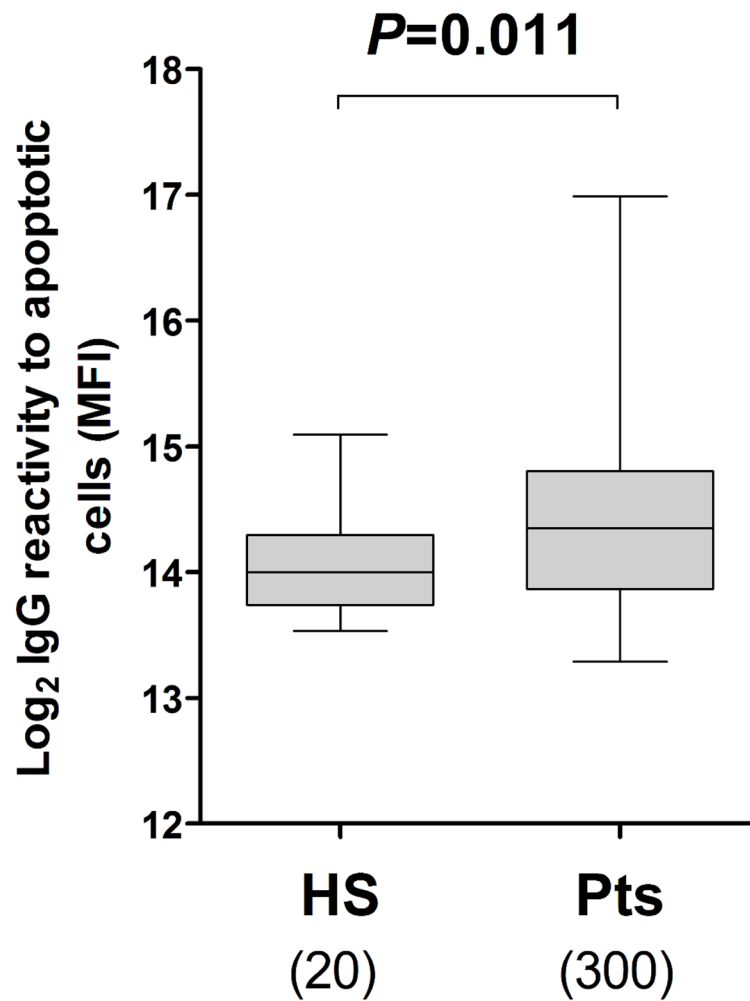


Figure 1. Pre-transplant purified IgG reactivity to apoptotic cells

The purified IgG reactivity to apoptotic Jurkat cells was measured by flow cytometry in samples collected pre-transplant from kidney transplant recipients as well as healthy subjects. Log₂ values of MFI are reported (y-axis). The numbers of samples tested in each group are shown below the box plot. The horizontal bar represents the median value; the bottom and top of each box represent the 25th and 75th percentiles; the lower and upper bars of each box represent the minimum and maximum values.

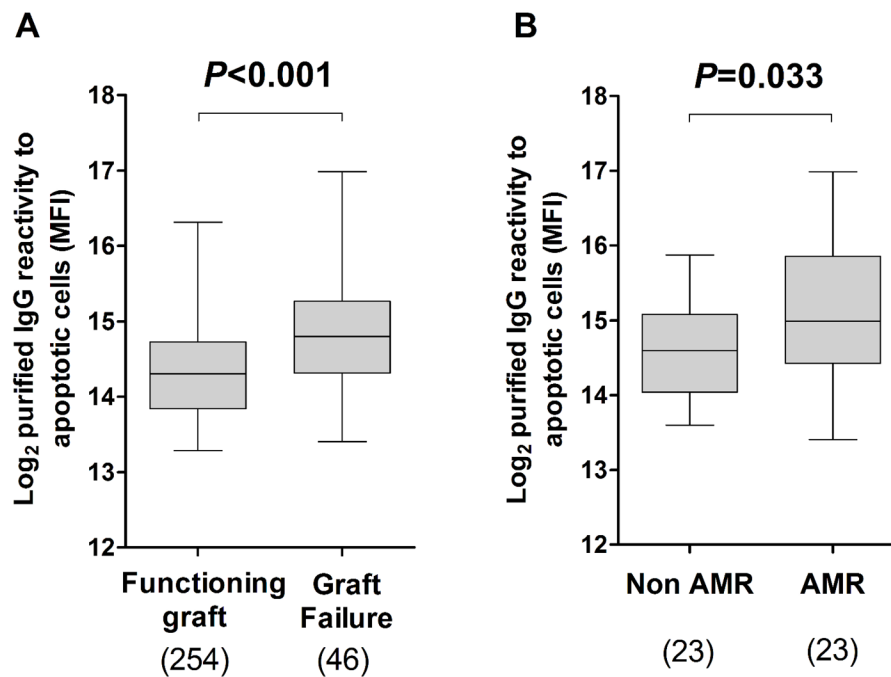


Figure 2. Pre-transplant purified IgG reactivity to apoptotic cells and graft loss

(A) Pre-transplant IgG reactivity to apoptotic cells was compared between patients with functioning graft and patients who experienced graft loss and (B) between patients whose graft loss was attributed to AMR and patients with other causes of graft loss. The numbers of samples analyzed in each group are shown below the box plot. The horizontal bar represents the median value; the bottom and top of each box represent the 25th and 75th percentiles; the lower and upper bars of each box represent the minimum and maximum values.

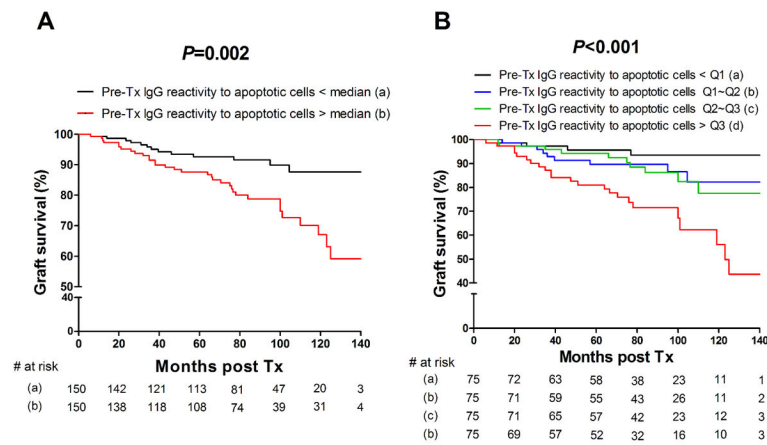


Figure 3. Pre-transplant purified IgG reactivity to apoptotic cells and graft outcome
Kaplan-Meier cumulative, death censored, graft survival plot analyzing the effect of pre-transplant purified IgG reactivity to apoptotic Jurkat cells (**A**) below (black line) or above (red line) the median value. (**B**) below the 1st quartile (black line), between the 1st quartile and the 2nd quartile (blue line), between the 2nd and 3rd quartile (green line) or above the 3rd quartile value (red line). The number of patients at risk is shown below each time point. P value among groups was computed using log-rank (Mantel-Cox) test.

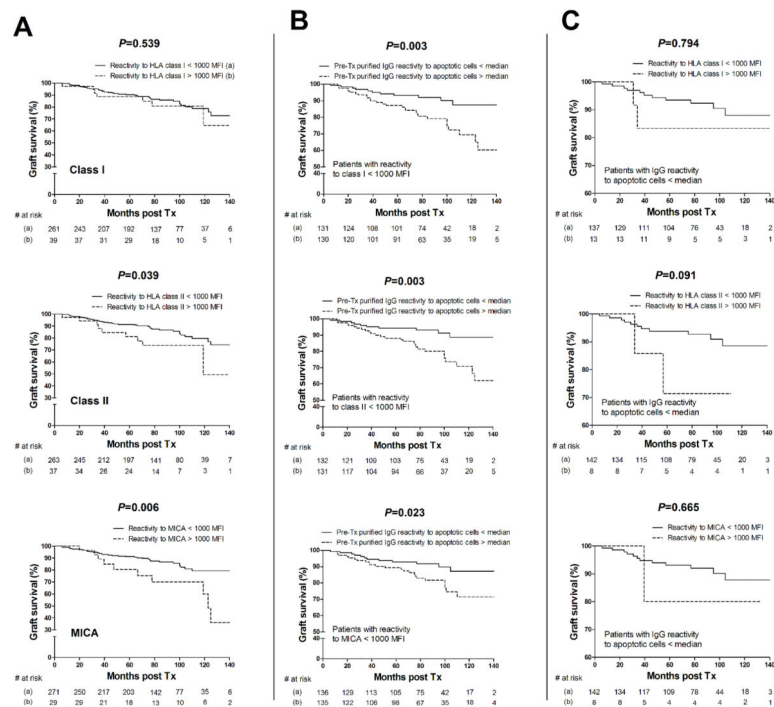


Figure 4. Kaplan-Meier cumulative graft survival

(A) Kaplan-Meier cumulative, death censored, graft survival plot analyzing the effect of pre-transplant IgG reactivity to HLA class I, HLA class II or MICA above (dot line) or below (solid line) a cutoff MFI value of 1000. (B) Kaplan-Meier cumulative, death censored, graft survival plot analyzing the effect of pre-transplant purified IgG reactivity to apoptotic Jurkat cells above (dotted line) or below (solid line) the median value after exclusion of patients with high reactivity to HLA class I, HLA class II or MICA (MFI > 1000). (C) Kaplan-Meier cumulative, death censored, graft survival plot analyzing the effect of pre-transplant IgG reactivity to HLA class I, HLA class II or MICA above (dot line) or below (solid line) a cutoff MFI value of 1000 after exclusion of patients with reactivity to apoptotic cells above the median value. The number of patients at risk is shown below each time point. *P* value between two groups was computed using log-rank (Mantel-Cox) test.

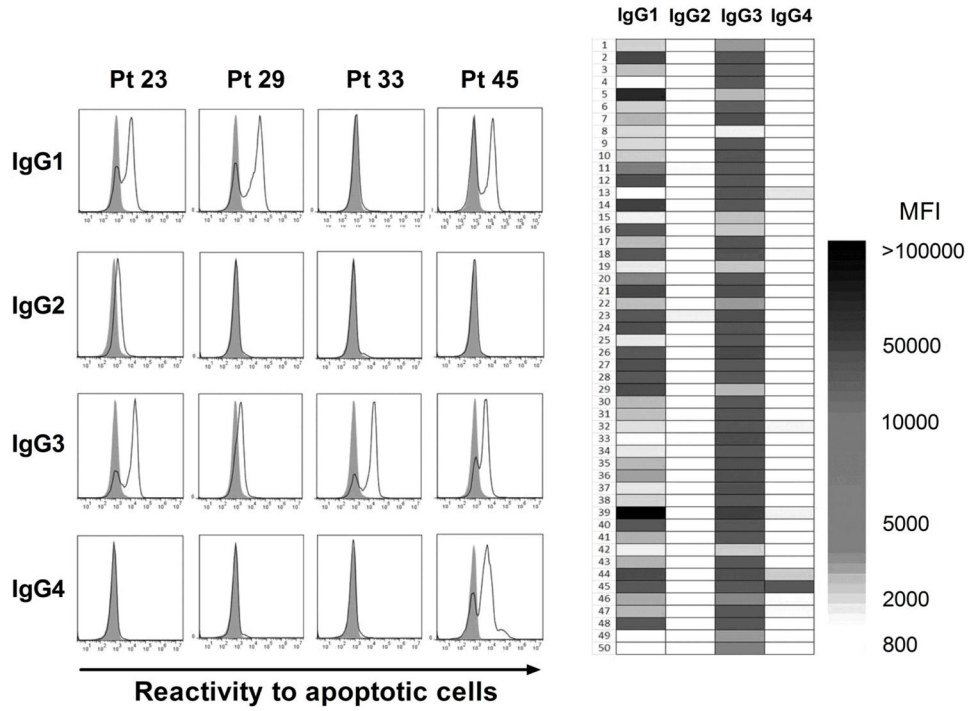


Figure 5. Subclasses of serum IgG reactive to apoptotic cells
(A) Example of subclass analysis by flow cytometry of IgG reactive to apoptotic Jurkat cells purified from 4 representative pre-transplant serum specimens, using secondary antibodies specific to IgG1 ~ IgG4. Filled gray histograms show background reactivity obtained with secondary antibodies alone. IgG1 ~ IgG4 reactivity to apoptotic Jurkat cells is depicted as solid line histograms. **(B)** Heat-map representation of IgG1 ~ IgG4 reactivity to apoptotic cells for the 50 most reactive serum samples. The shade of gray corresponds to the level of reactivity (MFI) as reported on the scale.

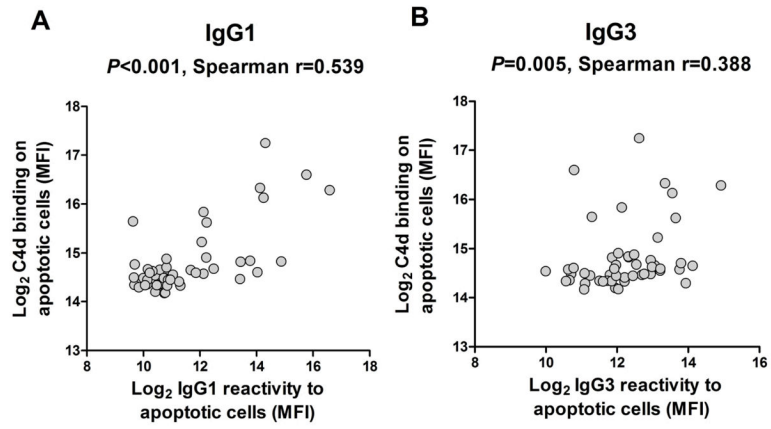


Figure 6. Correlation between complement activation and IgG subclasses reactivity to apoptotic cells

The deposition of C4d on the surface of apoptotic Jurkat cells was detected by flow cytometry after complement activation in vitro with IgG purified from the 50 most reactive pre-transplant serum samples. C4d deposition is reported together with IgG1 (A) or IgG3 (B) reactivity to apoptotic cells measured in the same samples.

Table 1

Patient characteristics

Parameters	Values
Age in year (mean±SD)	49.0±14.4
Sampling time pre-tx, month (mean±SD)	3.7±2.5
Follow-up, month (mean±SD)	81.2 ±35.3
Gender, n (%)	
Male	192 (64.0%)
Female	108 (36.0%)
Race, n (%)	
Caucasian	239 (79.7%)
African	29 (9.6%)
Asian	14 (4.7%)
Hispanic	18 (6.0%)
No. of transplant, n (%)	
First	259 (86.3%)
Second	35 (11.7 %)
Third	6 (2.0%)
Donation, n (%)	
Deceased	147 (49.0%)
Living related	84 (28.0%)
Living unrelated	69 (23.0%)
Transfusion pre-Tx, (n %)	
Yes	94 (31.3%)
No	206 (68.7%)
Induction therapy, (n %)	
Yes	84 (27.9%)
No	216 (72.1%)
Cause of end-stage renal disease, n (%)	
Polycystic kidney	36 (12.0%)
Focal Segmental Glomerulosclerosis (Primary)	26 (8.7%)
IgA nephropathy	39 (13.0%)
Type I diabetes mellitus	28 (9.3%)
Hypertension	21 (7.0%)
Obstructive/reflux uropathy/anatomical issues	22 (7.3%)
Systemic lupus erythematosus	6 (2.0%)
Immune complex disease	29 (9.7%)
Congenital/hereditary disease	18 (6.0%)
Interstitial nephritis	23 (7.7%)
Focal Segmental Glomerulosclerosis (Secondary)	12 (4.0%)
Type II diabetes mellitus	25 (8.3%)
Others	4 (1.3%)

Parameters	Values
Unknow	11 (3.7%)

Table 2

Cause of graft loss (N=46)

Cause of graft loss	Number
Antibody mediated rejection	17
Antibody mediated rejection & Cellular rejection	6
Cellular rejection	4
BK nephropathy	5
Recurrent IgA nephropathy	3
Recurrent FSGS	4
Transplant glomerulopathy	7

Table 3

Cox proportional hazards model. Hazard ratio (HR) with 95% confidence interval (95% CI) for graft survival in accordance with different variables

Variable	Hazard ratio	95% confidence interval	p-value
IgG reactivity to apoptotic cells ^a	2.271	1.530~3.369	<0.001
IgG reactivity to HLA class I ^a	0.909	0.739~1.118	0.366
IgG reactivity to HLA class II ^a	1.212	0.979~1.501	0.077
IgG reactivity to MICA ^a	1.015	0.863~1.195	0.857
Sex (male vs female)	0.964	0.499~1.865	0.915
African indicator	1.209	0.481~3.040	0.687
Asians indicator	0.600	0.079~4.539	0.621
Hispanic indicator	2.062	0.683~6.220	0.199
Age	0.996	0.972~1.019	0.715
cPRA	0.993	0.970~1.017	0.574
DGF	1.981	0.851~4.611	0.113
HLA mismatch	1.227	0.977~1.541	0.079
Cold ischemia time	1.000	0.947~1.057	0.994
Induction therapy	0.692	0.326~1.470	0.338
Living related donor	0.659	0.205~2.122	0.484
Living unrelated donor	1.029	0.372~2.846	0.956

^a per unit increase in $\log_2(1+\text{IgG reactivity})$